

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Wagle N, Grabiner BC, Van Allen EM, et al. Response and acquired resistance to everolimus in anaplastic thyroid cancer. *N Engl J Med* 2014;371:1426-33. DOI: 10.1056/NEJMoa1403352

## **SUPPLEMENTARY APPENDIX**

**Response and acquired resistance to everolimus in anaplastic thyroid carcinoma.** Wagle N, Grabiner, BC, Van Allen EM, et al.

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## **SUPPLEMENTARY METHODS**

### ***Patient and Tumor Samples***

All patients provided written informed consent for research biopsies and genomic profiling of tumor and normal DNA, as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol 11-104). A blood sample was obtained shortly after the initiation of everolimus, and whole blood was stored at -80°C until DNA extraction was performed.

### ***Histology and Staining***

Eight micrometer paraffin sections were deparaffinized with xylene and alcohol series, treated with Target Retrieval Solution pH 6.1 (Dako, Carpinteria, CA), blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol, and then put in 5% normal goat serum in 0.1% Triton X in PBS. Sections were incubated with primary antibodies overnight at 4°C, washed, and incubated with secondary antibody conjugated with horseradish peroxidase (HRP). DAB Chromogen solution (Envision+System Dako) was then applied to generate a color reaction. Slides were then counterstained with hematoxylin (Dako). Antibodies used for staining were: anti-pS6 (S235/236) (1:100, Cell Signaling), anti-TSC2 (1:100, Cell Signaling, Beverly, MA), anti-thyroglobulin NB100-65184 (1:1500, Novus), and anti-TTF1 M3575 (1:300, Dako)

### ***Whole Exome Sequencing***

*DNA extraction:* DNA extraction was performed as previously described<sup>1</sup>.

*Library Construction:* DNA libraries for massively parallel sequencing were generated as previously described<sup>1</sup> with the following modifications: the initial genomic DNA input into the shearing step was reduced from 3μg to 10-100ng in 50μL of solution. For adapter ligation, Illumina paired-end adapters were replaced with palindromic forked adapters (purchased from Integrated DNA Technologies) with unique 8 base index molecular barcode sequences included in the adapter sequence to facilitate downstream pooling. With the exception of the palindromic forked adapters, all reagents used for end repair, A-base addition, adapter ligation, and library enrichment PCR were purchased from KAPA Biosciences in 96-reaction kits. In addition, during the post-enrichment solid phase reversible immobilization (SPRI) bead cleanup, elution volume was reduced to 20μL to maximize library concentration, and a vortexing step was added to maximize the amount of template eluted from the beads. Libraries with concentrations above 40ng/μl, as measured by a PicoGreen assay automated on an Agilent Bravo instrument, were considered acceptable for hybrid selection and sequencing.

*Solution-phase hybrid selection:* The exon capture procedure was performed as previously described<sup>1</sup> with the following modifications: prior to hybridization, any libraries with concentrations >60ng/μL (as determined by PicoGreen) were brought to 60ng/μL, and 8.3μL of library was combined with blocking agent, bait, and hybridization buffer. Libraries with concentrations between 50 and 60ng/μL were normalized to 50ng/μL, and 10.3μL of library was combined with blocking agent, bait, and hybridization buffer. Libraries with concentrations between 40 and

50ng/ $\mu$ L were normalized to 40ng/ $\mu$ L, and 12.3 $\mu$ L of library was combined with blocking agent, bait, and hybridization buffer. Finally, the hybridization reaction was reduced to 17 hours, with no changes to the subsequent capture protocol.

*Preparation of libraries for cluster amplification and sequencing:* After post-capture enrichment, libraries were quantified using PicoGreen, normalized to equal concentration using a Perkin Elmer MiniJanus instrument, and pooled by equal volume on the Agilent Bravo platform. Library pools were then quantified using quantitative PCR (KAPA Biosystems) with probes specific to the ends of the adapters; this assay was automated using Agilent's Bravo liquid handling platform. Based on qPCR quantification, libraries were brought to 2nM and denatured using 0.2 N NaOH on the Perkin-Elmer MiniJanus. After denaturation, libraries were diluted to 20pM using hybridization buffer purchased from Illumina.

*Cluster amplification and sequencing:* Cluster amplification of denatured templates was performed according to the manufacturer's protocol (Illumina). HiSeq v3 cluster chemistry and flowcells, as well as Illumina's Multiplexing Sequencing Primer Kit. DNAs were added to flowcells and sequenced using the HiSeq 2000 v3 Sequencing-by-Synthesis method, then analyzed using RTA v.1.12.4.2 or later. Each pool of whole exome libraries was subjected to paired 76bp runs. An 8-base index sequencing read was performed to read molecular indices, across the number of lanes needed to meet coverage for all libraries in the pool.

*Sequence data processing:* Exome sequence data processing was performed using established analytical pipelines at the Broad Institute. A BAM file was produced with the Picard pipeline (<http://picard.sourceforge.net/>), which aligns the tumor and normal sequences to the hg19 human genome build using Illumina sequencing reads. The BAM was uploaded into the Firehose pipeline (<http://www.broadinstitute.org/cancer/cga/Firehose>), which manages input and output files to be executed by GenePattern<sup>2</sup>.

*Sequencing quality control:* Quality control modules within Firehose were applied to all sequencing data for comparison of the origin for tumor and normal genotypes and to assess fingerprinting concordance. Cross-contamination of samples was estimated using ContEst<sup>3</sup>.

### ***Somatic Alteration Assessment***

MuTect<sup>4</sup> was applied to identify somatic single-nucleotide variants. Indelocator (<http://www.broadinstitute.org/cancer/cga/indelocator>) was applied to identify small insertions or deletions. Artifacts introduced by DNA oxidation during sequencing were computationally removed using a filter-based method<sup>5</sup>. Annotation of identified variants was done using Oncotator (<http://www.broadinstitute.org/cancer/cga/oncotator>). Copy ratios were calculated for each captured target by dividing the tumor coverage by the median coverage obtained in a set of reference normal samples. The resulting copy ratios were segmented using the circular binary segmentation algorithm<sup>6</sup>. Genes in copy

ratio regions with segment means of greater than  $\log_2(4)$  were evaluated for focal amplifications, and genes in regions with segment means of less than  $\log_2(0.5)$  were evaluated for deletions.

### ***Cancer Cell Fraction Analysis***

Genome wide copy-ratios were estimated from whole-exome sequencing (WES) data by comparison of the observed depth of coverage at each exon to that achieved in normal samples. Allelic copy-ratios were then estimated by analysis of allelic fractions for all heterozygous SNPs identified in the paired normal sample. These allelic copy-ratios were then analyzed using ABSOLUTE<sup>7</sup> to generate cancer cell fractions as previously described.

### ***Plasmids and Site-directed Mutagenesis***

cDNA for wild type mTOR (pCDNA3-Flag-mTOR) was obtained from Addgene. The F2108L mTOR point mutation was generated in the parental vector using site-directed mutagenesis with the QuikChange II XL kit (Stratagene) according to the manufacturer's protocol using the following primers: F2108L\_TOP-5'-GGACCTCTATTATCATGTGTTACGACGAATCTCAAAGCA-3' and F2108L\_BOTTOM-5'-TGCTTTGAGATTCGTCGTAACACATGATAATAGAGGTCC-3'. The mutation was verified using Sanger sequencing as conducted by Eton Biosciences. WT and F2108L mTOR cDNAs were subcloned directly from the pcDNA3 vector into the pcDNA5/FRT/TO vector using single site NotI restriction digestion followed by ligation.

### ***Cell lines***

HEK-293T-TREX cells (Invitrogen) were maintained in DMEM with 10% heat-inactivated fetal bovine serum, and grown in a humidified incubator at 37°C, 5% CO<sub>2</sub>.

### ***Stable Transfections***

The HEK-293T-TREX cells were generated according to the manufacturer's protocol. Briefly, parental TREX cells were co-transfected with pCDNA5-mTOR cDNAs and pOG44 (FLP-expressing vector) using Fugene HD (Promega). 24 hours after transfection, the cells were plated at low density in hygromycin-containing media. Single cell clones were isolated, treated with doxycycline, and analyzed by Western blot for the presence of the Flag-tagged mTOR constructs.

### ***In vitro pharmacologic growth inhibition assays***

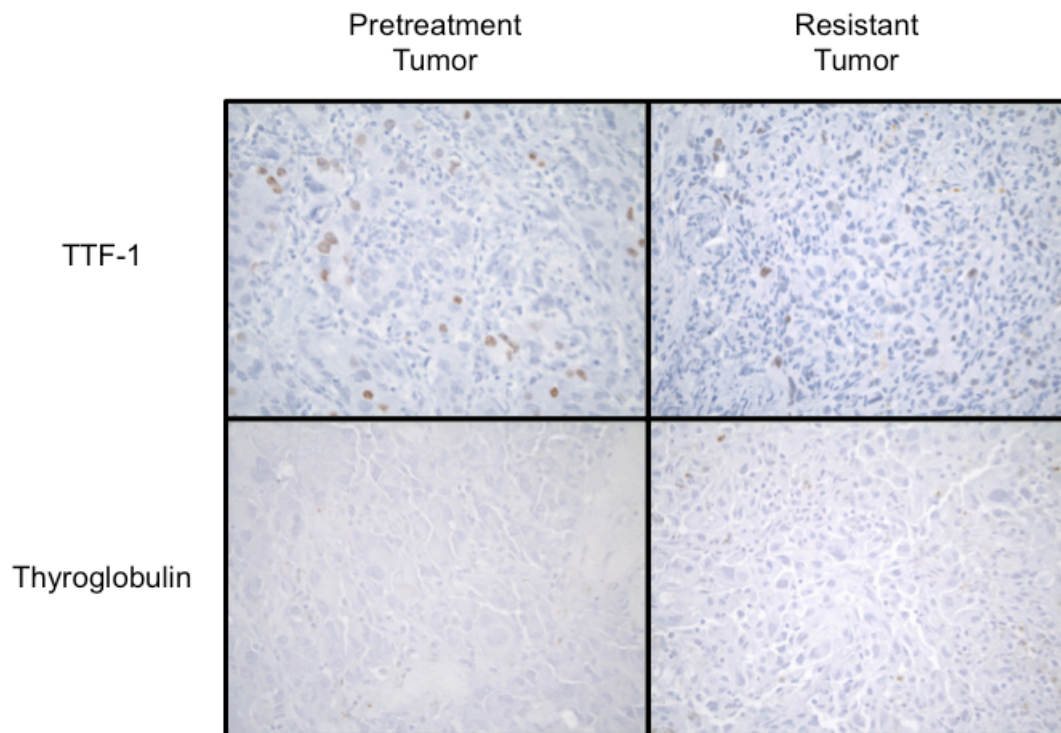
Six replicates each of 1000 cells were seeded into 96-well plates, treated with 8 doses of rapamycin or Torin1 24 hours later, and assayed for ATP content using Cell Titer Glo (Promega) 96 hours following drug treatment according to the manufacturer's protocol. Rapamycin was obtained from LC labs; Torin1 was generously provided by the Gray laboratory.

### ***Western blot analysis***

5x10<sup>5</sup> TREX cells per well were seeded into 6-well tissue culture plates, treated 24 hours later with rapamycin or Torin1 at the indicated doses for 1 hour, followed by whole cell lysis in NP-40 lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40 substitute, 1 mM EDTA at pH8.0, 50 mM NaF, 10 mM Na-pyrophosphate, 15 mM Na<sub>3</sub>VO<sub>4</sub>, 100 mM b-glycerophosphate, and 1 tablet of protease inhibitor (Roche Applied Science) per 50 mL of buffer). Whole cell lysates were mixed with 5x SDS-sample buffer to a final concentration of 2 mg/mL, boiled for 5 minutes, and then used directly for immunoblotting. 20 mg of whole cell lysates were loaded into lanes of 4-12% bis-tris SDS-PAGE gels (Invitrogen), run at 120 volts for 2 hours in 1x MOPS buffer (Invitrogen), and then transferred to 0.45 mm PVDF membrane (Millipore) at 130 mA for 2 hours in 1x transfer buffer (100 mM CAPS, 123 mM NaOH, 10% Ethanol). The membranes were then immunoblotted for the indicated proteins. Antibodies to S6K1 (clone 49D7) and phospho-T389-S6K1 (clone 108D2) were from Cell Signaling Technology; antibody to GAPDH (clone GT239) was from Genetex. Horseradish-peroxidase-labeled anti-rabbit secondary antibodies was from Santa Cruz Biotechnology. All primary antibodies were diluted 1:1000 in 5% BSA W/V TBST. All secondary antibodies were diluted 1:5000 in 5% milk W/V TBST. X-ray film was from Millipore and enhanced chemiluminescence reagent was from Thermo.

## SUPPLEMENTARY FIGURES

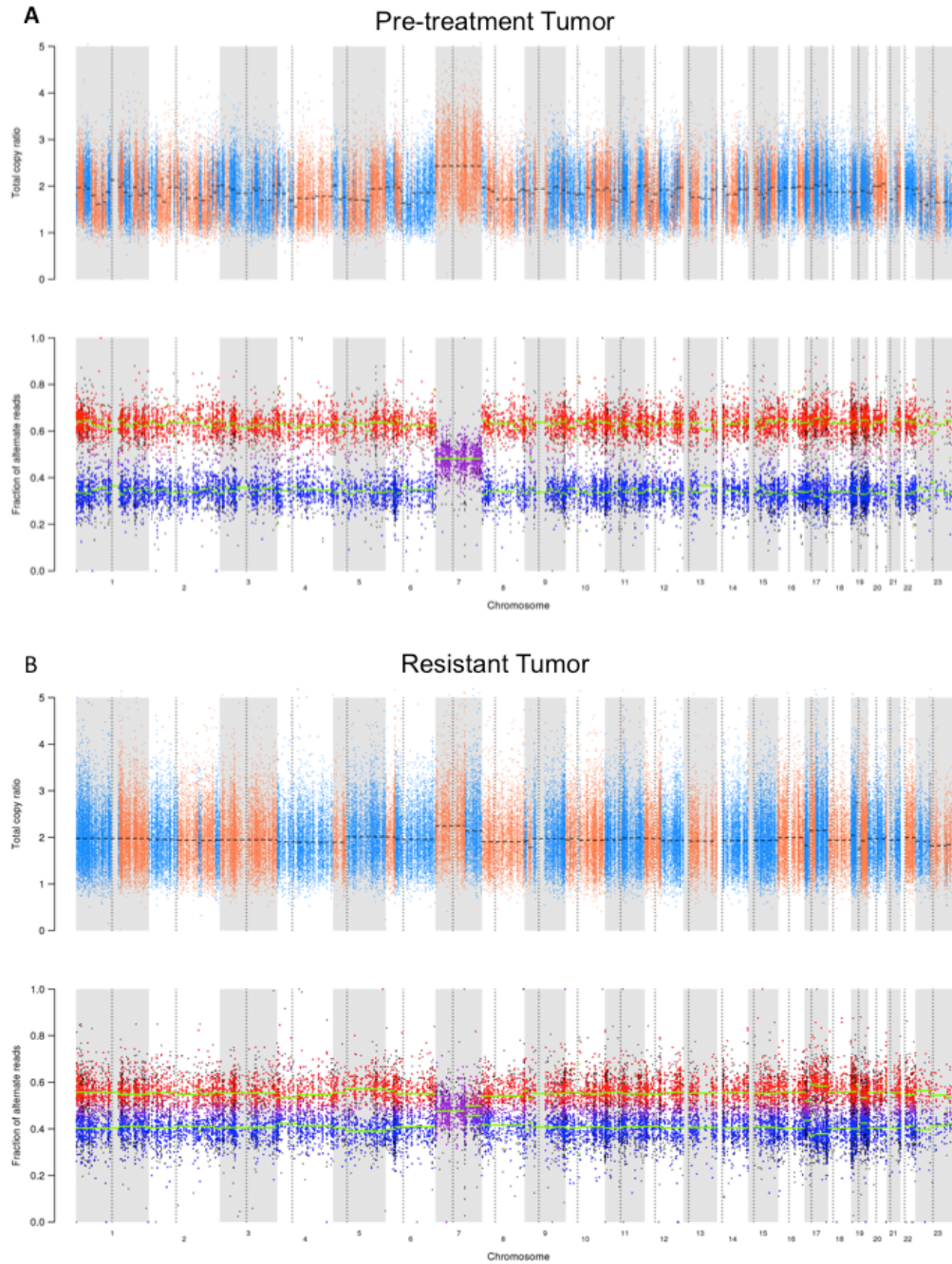
**Figure S1**



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**FIGURE S1. Immunostaining for TTF-1 and thyroglobulin in the pre-treatment and resistant tumors.** Immunostaining for TTF-1 and thyroglobulin in the pre-treatment and resistant tumor biopsies.

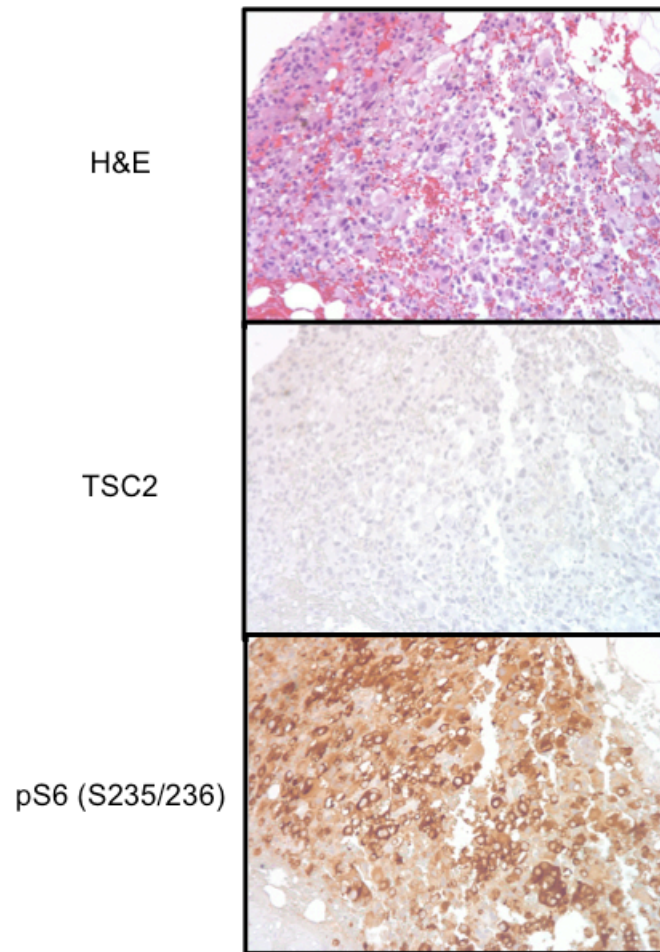
**Figure S2**



**FIGURE S2. Near haploidization of pre-treatment and resistant tumor genomes.**

Normalized total copy ratio and fraction of alternate reads at heterozygous sites (identified by UnifiedGenotyper<sup>8</sup>) for the pre-treatment tumor (Panel A) and resistant tumor (Panel B)<sup>7</sup>. The allele imbalance of heterozygous sites (expected diploid allele fraction of 0.5) across all chromosomes except for chromosome seven suggests that only one of the alleles at those sites is retained. This, along with a consistent copy ratio of 2 across the genome, supports a haploid genome with chromosome 7 retention.

**Figure S3**



**FIGURE S3. Immunostaining for TSC2 and phosphorylated S6K in the everolimus resistant tumor.** Histology of the anaplastic thyroid carcinoma in the post-treatment resistant tumor biopsy, with hematoxylin and eosin staining, TSC2 immunostaining, and immunostaining of phosphorylated S6, a downstream target of mTOR.

## **SUPPLEMENTARY TABLE**

### **TABLE S1. Sequencing Data and Analysis**

*“Supplementary Table 1 – Sequencing Data and Analysis.xlsx”*

- Tab 1: Coverage and Metrics
- Tab 2: Annotated SNVs and indels in the pre-treatment sample
- Tab 3: Annotated SNVs and indels in the resistant sample
- Tab 4: Comparison of cancer cell fractions (CCF) for SNVs and indels prior to clustering<sup>7</sup>
- Tab 5: Comparison of cancer cell fractions (CCF) for SNVs and indels after clustering<sup>7</sup>

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